# Natural killer cells in multiple sclerosis

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Invited Review

# Natural killer cells in multiple sclerosis: A review

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ABSTRACT

As the most common non-traumatic disabling disease among adolescents, multiple sclerosis (MS) is a devastating neurological inflammatory disease of the central nervous system. Research has not yet fully elucidated its pathogenesis, but it has shown MS to be a complex, multifactorial disease with many interplaying factors. One of these factors, natural killer (NK) cells, lymphocytes of the innate immune system, have recently gained attention due to the effects of daclizumab therapy, causing an expansion of the immunoregulatory subset of NK cells. Since then, NK cells and their relation to MS have been the focus of research, with many new findings being published in the last decade. In this review, NK cells are pictured as potent cytotoxic killers, as well as unique immune-regulators. Additionally, an overview of our current knowledge regarding NK cells in MS is given. The role of NK cells in MS is reviewed in the context of well-established environmental factors and current disease modifying therapies to gain further understanding of the pathogenesis and treatment options in MS.

## 1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory disorder resulting in demyelination and destruction of neurons in the central nervous system (CNS) [1]. In its most common phenotype, the relapsing-remitting (RR) variant (~85 %) [2], patients experience periods of neurological disability, like sensory or motor loss of a limb, followed by (sometimes only partial) recovery [3]. Although the exact mechanism of the development and the progression of MS is still unknown, many factors which contribute to its pathophysiology have been identified [4].

The view of MS on an immunological level has expanded and changed greatly over the years. Whilst classically viewed as a T helper-1 (Th-1) cell mediated disease, [5] many cells have since been identified as contributors to the disease. Notably, Th-17 [5,6], $CD8^+$ T-cells [7,8] and B-cells [7,9] are now generally considered to be involved in the inflammatory mechanisms of MS and treatments focussed on B-cells have shown positive results [10]. More recently, the natural killer (NK) cell has emerged as a contributor to the disease. NK cells are lymphocytes of the innate immune system that play a pivotal role in the defence against malignancies as well as viral infections. The identification of this new player in the field was mainly due to the treatment with daclizumab, an IL-2 receptor alpha chain (IL-2Rα; CD25) blocking monoclonal antibody that showed positive results in MS, potentially due to its effects on NK cells [11–13].

The dysfunctions in the immune system of MS patients are the result of the interplay between genetic and environmental risk factors. Genome wide association studies have identified a wide array of genetic polymorphisms linked to the immune system as risk-alleles for MS [14]. Environmental factors include vitamin D [15,16], viral infections like Epstein-Barr virus (EBV) [17] and cytomegalovirus (CMV) [18], smoking [19] and adolescent obesity [19,20]. The full effects of vitamin D on the immune system are not yet fully understood. Increasing evidence points towards a role in maintaining and restoring immune homeostasis and thereby a protective effect in MS [21,22]. This is considered to be important both in the onset of disease as well as in severity and progression [23]. The EBV hypothesis, postulating a dysfunctional or disproportional reaction to EBV infection as the cause for MS, is currently one of the best fitting models for the pathogenesis of MS. The theory classically claims a 'molecular mimicry' model as explanation for the auto-immune reaction. Recently, some other studies have postulated a model where immortalized B-cells, infected with EBV, play a role in priming and activating lymphocytes in tertiary lymphoid follicles in the meninges [24]. Levels of anti-EBV nuclear antigen 1 (anti-EBNA1) and anti-EBV viral capsid antigen (anti-VCA), which are an indication of EBV activity, are also linked to higher MS

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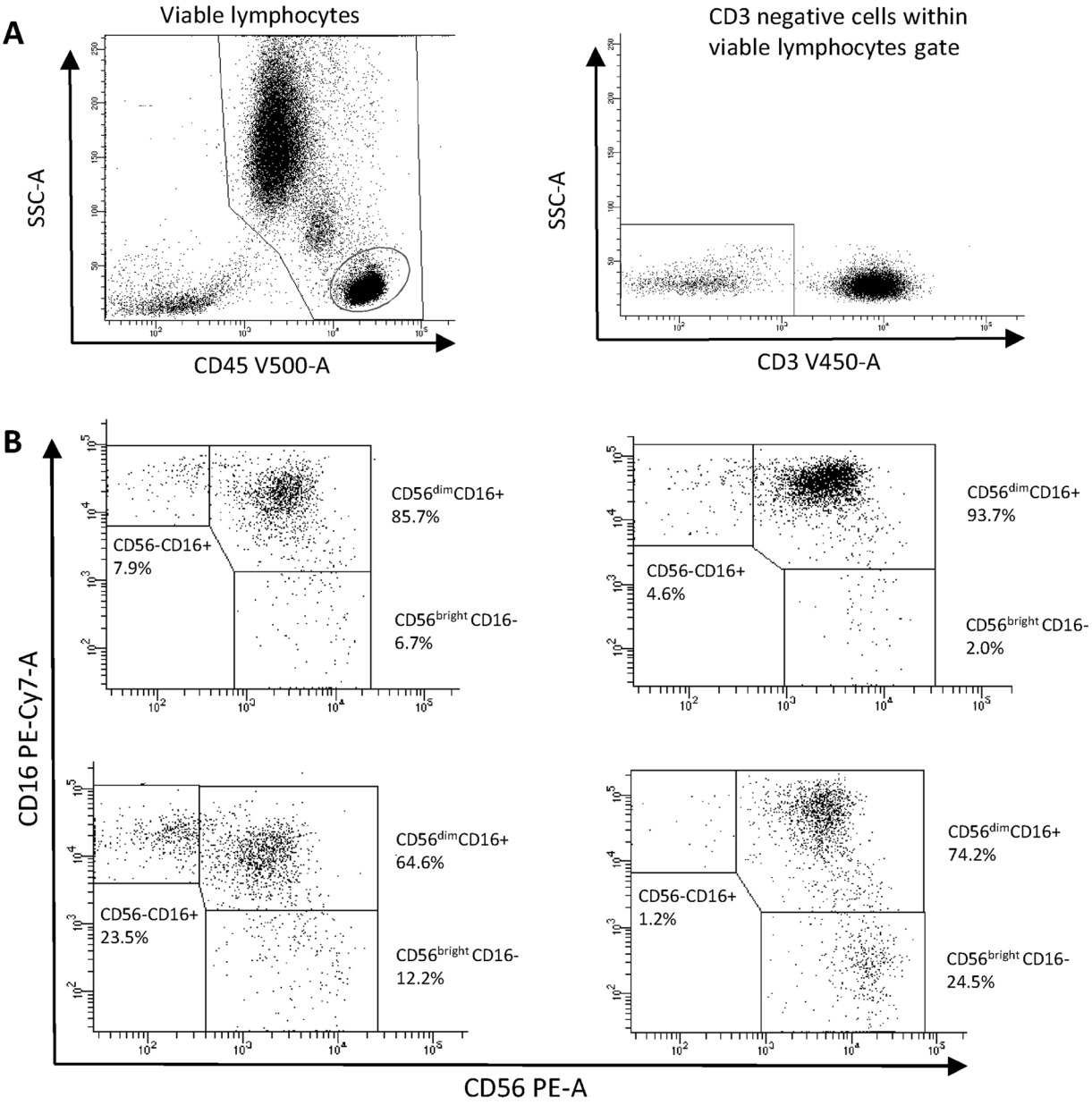


**Fig. 1.** Flowcytometry imaging of NK cells. Pictured is a step-by-step process to identify NK cells and their subsets. Aa. CD45 is used to find living cells. Then, the lymphocyte population can be defined after removing doublets using forward and side scatter. Using CD3, we can then differentiate T cells from B cells and NK cells. From the CD3 negative population, CD56-CD16- (B cells) are removed. The result is a population of only NK cells. **B**. NK cells can be subdivided in 3 subtypes based on their expression of CD56 and CD16. Although $CD56^{dim}CD16+$ and CD56-CD16+ are two different populations, they are functionally considered the same subtype. Also depicted are relative proportions of subtypes within the NK population. NK cell subpopulations of 4 patients are shown to illustrate the interpersonal variance in circulating NK cell phenotypes. Cy: cyanin; PE: phycoerythrin; SSC: side scatter.

disease activity [17]. Smoking and obesity are regarded as general inducers of an inflammatory state, thereby potentially contributing to many auto-immune diseases, including MS [19,20].

In this review, the associations between NK cells and MS and its relation with environmental factors are explored. Additionally, the effects of known MS therapies on NK cells are listed.

## 2. Natural killer cells

### 2.1. Subsets and functions

Human NK cells in peripheral blood are phenotypically defined as lymphocytes that lack the expression of CD3, but differentially express CD16 and CD56. Fig. 1 shows a main gating strategy for NK cells from PBMCs. Circulating NK cells can be divided into a $CD56^{bright}$ and $CD56^{dim}$ subset. Some, but not all patients also show a $CD56^{-}CD16^{+}$ phenotype, but generally this subset is pooled with the $CD56^{dim}$ subset as they seem to fulfil the same role in immunity [25]. Fig. 1 shows 4 different distributions of the NK cell compartment in order to showcase the interindividual variations of NK cells between patients. A further division in NK cell subsets can be made, based on several effector properties of these NK cells. Cichocki et al. divided the NK cell population into 4 subsets, based on their phenotype, *i.e.*, CD56 and CD16, migratory function and memory-like function [26]. The discriminating characteristics of these subsets are further described below.

The circulating $CD56^{bright}$ NK cells are immunoregulatory in nature through their cytokine production in response to chemical signalling [27]. Additionally, $CD56^{bright}$ NK cells produce granzyme K used to kill

**Table 1**
Activating and inhibitory human natural killer cell receptors.

| Receptor | Risk allele | Ligand | Risk allele | Receptor role | Comments |
|---|---|---|---|---|---|
| KIR-family | | | | | |
| KIR2DL1 | | HLA-C2 | | Inhibiting | During CNS inflammation, HLA class I (-related) molecules are more abundant due to damaged cells. The subtype of HLA class I (-related) molecule is dependent on genetic predisposition. |
| KIR2DL2/3 | | HLA-C1 | | Inhibiting | |
| KIR2DL4 | | HLA-G | | Activating | |
| KIR2DL5 | | ? | | Inhibiting | |
| KIR3DL1 | | HLA-Bw4 | | Inhibiting | |
| KIR3DL2 | | HLA-A3, -A11 | HLA-A | Inhibiting | |
| KIR2DS1 | | HLA-C2 | | Activating | |
| KIR2DS2 | | HLA-C1 | | Activating | |
| KIR2DS3 | | ? | | Activating | |
| KIR2DS4 | | ? | | Activating | |
| KIR2DS5 | | ? | | Activating | |
| KIR3DS1 | | HLA-Bw4 | HLA-B | Activating | |
| CD94-NKG2 | | | | | |
| NKG2A | | HLA-E | | Inhibiting | |
| NKG2C | | HLA-E | | Activating | |
| NKG2E | | HLA-E | | Activating | |
| NKG2D | | MIC-A/-B, ULBP1/2/3/4 | MIC-A | Activating | |
| NCRs | | | | | |
| NKp30 | | BAT-3, HSPG, B7-H6 | | Activating | BAT-3 is upregulated in PPMS [54] |
| NKp44 | | Viral HA | | Activating | |
| NKp46 | | Viral HA, HSPG | | Activating | |
| NKp80 | | AICL | | Activating | |
| 2B4 | | CD48 | CD48 | Activating | CD48 is upregulated in EAE [49] |
| DNAM-1 | CD226 | PVR, CD112 | PVR | Activating | |
| TIGIT | | PVR, CD112 | PVR | Inhibiting | |
| TACTILE | | PVR, CD112 | PVR | Inhibiting | |
| LILR | | HLA-I, UL18 | | Inhibiting | UL18 is related to CMV infection [52] |
| KLRG1 | | Cadherins | | Inhibiting | Certain cadherins are downregulated during exacerbations [50] |
| FcγRIII (CD16) | | Antibodies | | Activating | |
| IL-receptors | | IL | | | |
| IL-1R | | IL-1 | | Inhibitory | IL-1β is found in the blood, CSF and CNS lesions of MS patients [44] |
| IL-2R | IL2RA | IL-2 | | Activating | |
| IL-7R | IL7R | IL-7 | | Activating | In MS, IL-7 levels are reduced and IL-7Rα is increased [56] |
| IL-10R | IL10RB | IL-10 | | Inhibitory | IL-10 protects against TNF-induced relapses in EAE [46] |
| IL-12R | IL12RB1 | IL-12 | IL12A/IL12B | Activating | Levels of IL-12 are elevated in progressive MS patients serum [45] |
| IL-15R | IL15RA | IL-15 | | Activating | IL-15 is elevated in serum and CSF of MS patients [53] |
| IL-17R | | IL-17 | | Activating | IL-17 is significantly higher in MS patients [55] |
| IL-18R | | IL-18 | | Activating | IL-18 is increased in serum of MS patients; [51] IL-18R is upregulated in CSF of MS patients [48] |
| IL-21R | | IL-21 | | Activating | IL-21 is correlated with more severe MS disease course [47] |

Overview of activating and inhibitory receptors on NK cells with their corresponding ligands and function. AICL: activation-induced C-type lectin; BAT-3: HLA-B-associated transcript 3; DNAM-1: DNAX accessory molecule-1; HA: hemagglutinin; HLA: human leukocyte antigen; HSPG: heparan sulphate proteoglycan; IL: interleukin; IL-R: interleukin-receptor; KIR: killer-cell immunoglobulin-like receptor; KLR: killer cell lectin-like receptor; LILR: leukocyte immunoglobin-like receptor; MHC: major histocompatibility complex; MIC: MHC class I polypeptide-related sequence; NCR: natural cytotoxicity receptor; PVR: polio virus receptor; RAE-1: retinoic acid early transcript-1; TACTILE: T-cell activation, increased late expression, CD96; TIGIT: T cell immunoglobin and immunoreceptor tyrosine-based inhibitory motif domain); ULBP: UL16 binding protein. Adapted from: Pegram, H.J. et al., Activating and inhibitory receptors of natural killer cells. Immunol Cell Biol, 2011. 89(2): p.216-24.

activated $CD4^+$ T cells [28]. As $CD56^{bright}$ NK cells do not express receptors of the killer-cell immunoglobulin-like receptor (KIR) family, they are less prone to activation by cell-to-cell contact, but more sensitive to cytokine signalling by receptors like the IL-2R or IL-15R. In fact, $CD56^{bright}$ NK cells express high levels of the IL-2R, both as medium affinity (IL-2Rβγ) and high affinity (IL-2Rαβγ) receptors [29], ensuring its activity and survival in an environment with relatively low IL-2 levels. The cytokines produced by NK cells depend on the manner of activation of the cell. Co-stimulation of IL-12 and IL-18 efficiently induces production of IFN-γ, but not IL-10. On the other hand, IL-10 is produced upon stimulation of IL-12 together with IL-15 or IL-2 [30]. This specific reaction to different cytokine mixtures, combined with a sensitivity to many chemokines, makes the $CD56^{bright}$ NK cell a potent immunoregulatory cell which could play a protective role in auto-immunity.

Canonical $CD56^{dim}$ NK cells are the most abundant in peripheral blood and the most well-known subtype. These cells fulfil a role of immune-surveillance, in that they 'scan' cells for infection or malignant activity. Once a $CD56^{dim}$ NK cell is activated by a relative abundance of activating receptor stimulation, it utilises granzyme B and perforin to induce apoptosis in the target cell. The $CD56^{dim}$ NK cell also releases IFN-γ in response to 'finding' an unhealthy cell, thereby creating a pro-inflammatory environment to further combat the infection or malignant tumour [31]. This pro-inflammatory environment could contribute to the deterioration of auto-immune diseases, like exacerbations in MS, by triggering a re-activation of the (auto-)immune response. $CD56^{dim}$ NK cells also express CD16 (FcγRIII) which allows the NK cell to sense antibody coated cells and eliminate them through antibody dependent cellular cytotoxicity (ADCC), giving the $CD56^{dim}$ NK cells another potent mechanism of eliminating potential threats. It is important to note that, while $CD56^{bright}$ cells are primarily considered as cytokine producers and $CD56^{dim}$ cells as cytotoxic cells, both subtypes are capable of utilizing both mechanisms. The main difference between the subsets is not their mechanisms of action, but rather the means of activation, as $CD56^{bright}$ cells respond to soluble signalling, while $CD56^{dim}$ cells respond to cell-to-cell signalling.

The concept of a 'memory' CD56$^{dim}$ NK cell has been controversial, as memory is considered to be a defining and exclusive feature of the adaptive immune system. However, research in murine models from O'Leary et al. [32] demonstrated that NK cells can, in absence of T- and B-cells, mediate a contact hypersensitivity response, typical of a memory-mediated delayed reaction. Later, Cooper et al. [33] demonstrated that previously activated murine NK cells responded more robustly to stimulation by cytokines. Additionally, when transferring these activated NK cells to naïve donors, they started proliferating whilst maintaining their sensitivity to stimulation. This finding was supported by Sun et al. [34], who demonstrated a rapid expansion of murine NK cells bearing the Ly49H receptor, which specifically recognizes murine cytomegalovirus (CMV), after infection with MCMV. Again, transferring these NK cells to naïve mice and introducing a viral challenge resulted in a far more robust NK cell response and protective immunity. This memory-like phenomenon can also be found in human NK cells. Within the innate immune system, this memory-like process is called 'trained immunity' [35]. These memory-like NK cells are typically found in people infected with human CMV [36] and have several characterising properties. For one, an upregulation of the inhibitory NKG2C receptor was found, together with a downregulation of the FcεRI gamma chain adaptor, which is coupled to CD16 [36,37]. The subsequent replacement of the γ chain with the ζ chain is thought to enhance antibody-dependent NK cell activation [38,39]. As such, this subset seems to be less potent in its surveillance role, but far more potent in its antibody dependant role, particularly in cytokine production, compared to regular NK cells [40]. Another characteristic of memory-like NK cells includes the epigenetic silencing of promyelocytic leukemia zinc finger (PLZF) transcription factor, with a currently unknown consequence for the NK cell function [36,37]. The relation between MS and these memory-like NK cells has been investigated [41], but more research is necessary to gain a full understanding of the role memory-like NK cells may have in the inflammatory process of MS.

Tissue-resident NK cells are characterised by a lack of migratory receptors and thus a limited capacity to enter the blood stream. Tissue-resident NK cells have been found in the liver, spleen and uterus, each with different phenotypes [42], To the best of our knowledge, no study investigating a CNS specific NK cell subset has been performed. However, since tissue-resident NK cells are thought to play a role in immune homeostasis [43], a disturbance in this subset, even if not localized within the CNS, may alter the intrathecal immune response, thus affecting the course of MS. Obviously, since this remains mere speculation, research into this NK cell subset is warranted to elucidate its potential role in MS.

### 2.2. Activation and inhibition

As stated earlier, both CD56$^{bright}$ and CD56$^{dim}$ NK cells rely on a complex system of activation *versus* inhibition in order to fulfil their effector function. Many activating receptors and inhibitory receptors and their ligands have been identified, as listed in Table 1. [44–56] Table 1 also lists if the gene coding for the receptor/ligand has come up in GWAS studies, as well as their known up-/downregulation in MS.

For activating receptors, several have been extensively studied and their mechanism of action is relatively well known. Prime examples are natural killer gene 2D (NKG2D, CD314), 2B4 (CD244), the natural cytotoxicity receptors (NCR) NKp30, NKp44 and NKp46, and DNAM-1 (DNAX accessory molecule-1). While these receptors can be potent in activating NK cells, a combination of activating signals, like NKp46 with NKG2D, is always required in order to initiate activation [57]. This most likely constitutes a fail-safe mechanism to ensure NK cells do not kill healthy cells. Only signalling through CD16 can independently activate an NK cell. In this case, the specificity of the response is determined through B-cell activity, *i.e.*, antibody production, thus providing a reason why no co-stimulation is needed when circulating NK cells are activated through CD16.

Inhibitory receptors are less abundant than their activating counterparts, but play a more dominant role in the triggering of the NK cell [57]. Inhibitory receptors include members of the KIR family, as well as NKG2A, CD96 (TACTILE) and T-cell immunoreceptor with Ig and ITIM domains (TIGIT). The latter two are paired receptors with the activating DNAM-1 receptor. Interestingly, although many variations of KIR have been identified, a single NK cell expresses only a few [58]. This random selection of inhibiting receptors seems a way for NK cells to better detect different phenotypes of infected/tumour cells. KIR recognise self HLA-A, -B and -C molecules, while NKG2A recognises the less variable HLA-E molecules [59]. TIGIT, like DNAM-1, binds polio virus receptor (PVR) and nectin-2. When one of these inhibitory receptors is engaged, it seems that the activating signalling pathway is blocked, thus preventing the NK cell from initiating its effector function [60]. Even here, it seems that some signals are stronger than others, as it has been shown that NKG2D dependent signalling is easier to inhibit than ADCC signalling *via* CD16 [61]. Inhibitory receptors also seem to be involved in the 'licensing' of NK cells. This detailed mechanism is reviewed elsewhere [57] and is beyond the scope of this review.

## 3. Multiple sclerosis

### 3.1. Disease characteristics

MS is the most common non-traumatic disabling disease to affect young adults [62]. Its prevalence varies greatly, between 2 in 100.000 in Japan and greater than 200 in 100.000 in Northern Europe and North America [2], with its prevalence increasing with latitude [63]. There is also a gender bias, since women are more often affected compared to men (3:1) [64,65]. MS is most frequently diagnosed between the ages of 25–40, where a patient can present with a clinically isolated syndrome (CIS). This constitutes a mono- or poly-symptomatic event (depending on its corresponding lesion in the CNS) which develops acutely or subacutely and remains for at least 24 h [66]. Afterwards there may be a period of recovery, although this is not guaranteed. Typical first presentations include a unilateral optic neuritis (ON), partial myelopathy, focal supratentorial syndrome or focal brainstem or cerebellar syndrome [67]. Frequent symptoms in definite MS include fatigue, reduced walking range, paraesthesia, hypaesthesia, muscle weakness and imbalance [68]. An important diagnostic tool in MS is magnetic resonance imaging (MRI) of the CNS, where typical T2-hyperintense areas may be found around the ventricles (paraventricular), directly adjacent to the cortex (juxtacortical), in the cortex (cortical), infra-tentorial (brainstem/cerebellum) and in the spinal cord. The addition of gadolinium contrast may show contrast enhancement, indicating leakiness of the blood brain barrier due to lymphocyte trafficking. In order to provide a definite MS diagnosis, a dissemination in space (DIS) and dissemination in time (DIT) is required [66]. With a better use of MRI techniques, DIS and DIT are found much earlier and it is no longer required to wait for a second clinical relapse before starting treatment [67]. Additionally, the most recent revisions of the McDonald criteria allow presence of cerebrospinal unique oligoclonal bands to replace the DIT criterion [66]. RRMS is the most prevalent subtype of MS and the focus of almost all disease modifying therapies (DMTs) [2]. For most of these patients (65 % of all RRMS patients [69]), the disease later evolves into a steadily progressing variant, where relapses are replaced by gradual deterioration of the clinical condition. In more contemporary cohorts treated early with disease modifying therapies, a lower estimate of 15–30 % secondary progression has been reported [70]. This secondary progressive MS (SPMS) variant has a less diverse repertoire of treatment options, with currently only interferon-beta being approved for active SPMS treatment [71]. Additionally, an upcoming drug named siponimod is showing promising results in treatment of SPMS patients, reducing their rate of deterioration significantly [72]. Patients may also present with gradually progressive neurological deterioration, without ever experiencing relapses. These patients suffer

from a primary progressive MS (PPMS) and currently, only ocrelizumab (anti-CD20) is approved for treatment of PPMS [73]. This division into different subtypes is becoming increasingly controversial, as research shows little genetic and clinical distinguishing properties between the subtypes [74]. A more recent classification categorises MS as either relapsing-remitting or progressive. Progressive MS could then be characterized by both 'activity' and 'progression' [75]. Activity is described as the presence of clinical exacerbations, together with MRI findings. These are also the most common endpoints used in intervention trials for RRMS and are frequently reported as a measure of effectiveness of the drug. 'Progression' is monitored through yearly measurement of the expanded disability status scale [76] and implies a loss of function, regardless of new MRI lesions or exacerbations.

### 3.2. Natural Killer cells in multiple sclerosis

The role of NK cells in MS has been a controversial topic, with studies reporting both a protective and a damaging role in MS and experimental auto-immune encephalomyelitis (EAE, the induced variant of MS in animals, usually mice) [77–79]. Much of the exploratory research into NK cells is based on EAE models. Unfortunately, as murine NK cells do not express CD56 and express different receptors, the results of EAE studies are not easily extrapolated to human patients. There are, however, some similarities between murine and human NK cells. The murine NK cell population can be subdivided into $CD27^{high}$ and $CD27^{low/-}$, considered to be the equivalent of the human $CD56^{bright}$ and $CD56^{dim}$ NK population, respectively [80]. EAE is also different from MS in some aspects. For example, EAE is induced by immunisation with myelin peptides like myelin basic protein [81], even though the exact target or mechanism of auto-inflammation is unknown in MS. These differences can cause discrepancies between murine and human studies. Considering the difficulty of finding patients with MS before clinical manifestation and subsequent lack of understanding of MS pathogenesis, EAE is an excellent model to study the early, pre-clinical stage of MS. For example, one EAE study performed in mice found that in the pre-clinical stage of EAE, the murine NK cell compartment undergoes several changes. For one, The proportion $CD27^{low/-}$ NK cells increased. Additionally, the receptors of the (immunoregulatory) $CD27^{high}$ NK cell population shifted towards a more inhibitory phenotype, including downregulation of the activating Ly49D, Ly49H and NKG2D receptors [82]. Increasing our knowledge of immunological changes leading to clinical disease could increase our insight in MS pathogenesis and provide new therapeutic targets.

Enhancing the regulatory features of NK cells ameliorates the course of EAE. When blocking NKG2A and Qa-1 (the murine equivalent of HLA-E), NK cells inhibited CNS inflammation by killing T cells and microglial cells [83,84]. On the other hand, recent research shows that NK cells in MS may also contribute to CNS damage. According to Liu et al., in MS and EAE, NK cells are in contact with neural stem cells (NSCs) and, in EAE, NSCs release IL-15 upon contact with NK cells [85]. IL-15 in turn supports proliferation and survival of NK cells, thus contributing to a stronger NK cell response. However, particularly during the later stages of EAE, NK cells kill NSCs, mainly due to reduced expression of Qa-1. Removing NK cells during the later phases of EAE indeed ameliorated the disease severity [85]. Liu et al. did not determine which subset of NK cells was responsible for these observations. Other studies show that the NK cell population in the CNS and CSF consists mainly of $CD56^{bright}$ NK cells [86,87]. This is true for MS patients, but also healthy controls and patients with other neurological disorders, which points towards a location specific enrichment of $CD56^{bright}$ NK cells rather than a MS specific phenomenon [88]. This might be related to the higher migratory capacity past the blood brain barrier (BBB) of $CD56^{bright}$ NK cells compared to $CD56^{dim}$ NK cells [89]. Even though $CD56^{bright}$ NK cells are abundant in the CNS and CSF, this does not mean that they are responsible for the destructive findings as described by Liu et al. A post-mortem study shows that NK cells most likely damage the myelin in an antibody dependent mechanism, *i.e.*, ADCC, suggesting that $CD56^{dim}$ NK cells are responsible for the damage to the CNS [90]. More research is necessary to fully elucidate the mechanisms of action of both NK cell subsets in MS.

Given their immunoregulatory properties, $CD56^{bright}$ NK cells have been monitored in untreated MS patients, patients with clinically isolated syndrome (CIS) suggestive of MS, and healthy controls [91]. Interestingly, the number of $CD56^{bright}$ NK cells in peripheral blood is similar between MS patients and healthy controls. However, when stimulated with cytokines, the $CD56^{bright}$ population in MS patients was less efficient in killing activated T-cells than in healthy controls. This difference in efficiency was attributed to an increased expression of HLA-E on T-cells, thus providing more ligand for NKG2A, which acts as an inhibitory receptor on NK cells [91,92]. HLA-E is also found to be upregulated in MS patients in white matter lesions, endothelial cells and astrocytes [93]. Immune cells and neural cells in MS plaques also express higher levels of HLA-E [92]. This would imply that the reason for the weakened response is not a dysfunctional NK cell population, but rather a resistance of target cells towards NK cells due to HLA-E upregulation in MS.

The largest breakthrough for NK cells in MS came with the introduction of daclizumab [94]. This anti-CD25 antibody effectively blocks the α-component of the IL-2R, which is needed to turn a medium affinity IL-2R into a high-affinity IL-2R [95]. The intended mechanism of action of daclizumab was to inhibit T-cell activation, since these T-cells express the high-affinity IL-2R and require IL-2 for activation/survival [96]. An MS risk-allele can be found in the IL2RA gene (coding for CD25), thus supporting the theory of its involvement in MS [97]. Additionally, this IL2RA gene seems to be influenced by vitamin D in T-cells, thus providing another connection with known MS risk factors [98,99]. Early results showed positive outcomes using daclizumab in MS [89,100,101], but paradoxically later studies revealed that blocking IL-2R actually enhanced the T-cell response *in vitro*, instead of dampening it [102,103]. Additionally, blocking IL-2R meant that regulatory T-cells (Tregs), which normally suppress the immune response, were also inhibited. As such, blocking IL-2R seemed to be more pro- than anti-inflammatory by inducing T-cell immunity and dampening immune regulation by Tregs. Another mechanism of action was sought to explain the beneficial effect of daclizumab, which was found in the expansion of the $CD56^{bright}$ NK cell population, expressing the medium-affinity IL-2R. Because of the relative abundance of IL-2 due to blocking of the high affinity IL-2R, these cells could expand around 400–500 % [89,102]. Continuing this line of research, it was later found that $CD56^{bright}$ NK cells can kill (autologous) activated T-cells, thereby severely limiting the activity of the T-cell population [28,102]. The expansion of $CD56^{bright}$ NK cells apparently outweighed the reduction in Treg activity. This finding provided solid support for the immunoregulatory and protective role of $CD56^{bright}$ NK cells in MS. Indeed, one Australian study found an inverse correlation between MRI lesions and $CD56^{bright}$ NK cells [104].

## 4. Interaction with risk factors

Despite many genetic loci being associated with MS, genetic aberrations do not fully account for its pathogenesis and course. Only a fraction of the pathogenesis is explained by genetics (a maximum of 25 % concordance in twin studies [105]), of which nearly 50 % has been accounted for by recent work by the international MS genetic consortium [97]. Epidemiological data points towards a key role for environmental factors in MS. Of these factors, the ones that are currently the most impactful and best understood are vitamin D, infection with EBV and CMV, smoking and adolescent obesity. Full details on how these factors impact MS are beyond the scope of this review. However, looking at how these environmental factors interact with NK cells and *vice versa* could provide new insights into MS. As the interplay between the different immunological players and environmental factors is

becoming increasingly complex, this review focuses mainly on the direct effects of environmental factors on NK cells.

### 4.1. Vitamin D

When looking at the distribution of MS patients worldwide, an increase in prevalence is found in increasing latitudes [63,106]. Much of this effect is attributed to diminished sun-exposure in countries with higher latitudes, causing less vitamin D to be synthesised in the skin. Genetic pre-disposition did not seem related, as studies with adolescent migrants show a higher risk of developing MS for migrants who move from lower to higher latitudes. Conversely, adolescent migrants from higher latitudes have a lower chance of developing MS when moving towards lower latitudes [107]. Vitamin D is gained through sun-exposure and nutrition, but requires some activating steps in the body before its active variant can be created [108]. Most studies regarding MS, NK cells and vitamin D have used vitamin $D_3$ (cholecalciferol) supplementation. Usually, vitamin D levels are measured in 25-hydroxyvitamin D (calcifediol) which is the precursor for active vitamin D (1,25-dihydroxyvitamin D or calcitriol). Vitamin D is considered to support immune homeostasis [109,110], mainly through the suppression of pro-inflammatory cytokine production by effector T-cells [111]. Since NK cells can show gene expression for VDR [112], a direct effect of vitamin D is to be expected on NK cells as well. Indeed, treating NK cells with 1,25-dihydroxyvitamin D *in vitro* shows an increase in cytotoxicity without altering the proliferation of NK cells [113,114]. Several findings support the notion that vitamin D influences NK cell cytotoxicity. For example, studies including elderly patients show that those with low 1,25-dihydroxyvitamin D levels have higher circulating NK cell numbers, likely as a compensation for a reduced cytotoxic potential per individual NK cell [115,116]. Also, patients with chronic renal failure (who cannot produce sufficient 1,25-dihydroxyvitamin D) and vitamin D resistant rickets have an impaired immune response, including a decreased NK cell functionality, which can be restored by cholecalciferol or 1,25-dihydroxyvitamin D supplementation [117,118]. It is important to note, however, that adding 1,25-dihydroxyvitamin D in a PBMC culture reduces NK cell cytotoxicity. This has been attributed to the production of prostaglandins by monocytes, which express relatively more VDR and thus respond more strongly to vitamin D. Prostaglandins in turn reduce the effectiveness of NK cells [119,120]. As such, it seems more likely that a lack of vitamin D reduces the NK cell cytotoxicity, but an abundance of vitamin D does not cause the NK cell to become 'overactive'. Besides the influence on cytotoxicity, vitamin D seems to also influence other facets of the NK cell. Indeed, Weeres et al. point towards a role for 1,25-dihydroxyvitamin D in the developmental process of NK cells, showing a reduced population and reduced cytotoxicity *in vitro*, using HSC cultures treated with physiological 1,25-dihydroxyvitamin D levels [121]. They suggest an immunoregulatory role of 1,25-dihydroxyvitamin D by impacting the early development of NK cells at the level of HSCs, seemingly favouring a development of HSCs into monocytes instead, although they do not report on the effect of 1,25-dihydroxyvitamin D on the differentiation into different NK cell subsets. Another influence of vitamin D is reported by Olson et al., who focussed their research on large granular lymphocyte leukaemia. This rare cancer of the T-cell or NK cell lineage [122] is associated with EBV infection and characterised by hyperactivation of the 'signalling transcription and transduction' (STAT)-1, STAT-3 and STAT-5 pathway, resulting in overproduction of cytokines [123,124]. The group studied the effect of 1,25-dihydroxyvitamin D *in vitro* on the malignant, hyperactive NK cell functionality and cytokine production and found a decrease in STAT-activation and IFN-γ production under stimulation of the VDR, pointing towards a regulatory role for 1,25-dihydroxyvitamin D in NK cells. Despite the many promising results regarding the effects of 1,25-dihydroxyvitamin D, it remains unclear whether the supplementation of 25-hydroxyvitamin D actually alters the NK cell population at any level. As such, future research on this subject should be focussed on the effect of supplementing cholecalciferol *in vivo* and monitoring possible effects on the NK cell population, NK cell subsets and their cytokine panel in MS patients. Nevertheless, these studies do provide evidence of 1,25-dihydroxyvitamin D interacting with NK cells and may offer new insights into the link between vitamin D and the pathogenesis and course of MS.

### 4.2. Viral infections

EBV is a virus of the Herpes family, which mainly transmits through contact with saliva of infected individuals [125]. EBV infects epithelial cells and B-cells, after which it persists in B-cells for the rest of the individuals life. EBV usually persists asymptomatically in healthy and immune-competent individuals and around 90 % of the worldwide population shows signs of EBV exposure. Generally, EBV infection happens during early childhood and is asymptomatic. However, EBV infection in adolescence/adulthood may manifest as infectious mononucleosis (IM) in 30–40 % of patients, associated with chronic fatigue and lymphadenopathy [126]. Its association with MS is evident when looking at epidemiological studies. Of all MS patients, over 99 % show signs of EBV exposure and a medical history of IM increases the risk of developing MS about two-fold compared to overall EBV exposed individuals [127–129]. Moreover, more recent evidence leads some authors to question if a truly EBV-seronegative MS patient even exists, further implying EBV's crucial role in MS pathogenesis [130,131]. The exact mechanism through which EBV contributes to the pathogenesis of MS remains unclear, although a few hypotheses have been formulated, including molecular mimicry and B-cell immortalisation. In almost every case, a role is reserved for the immortalised EBV-infected B-cell, which would normally be kept under strict control by the healthy immune system [132]. Markers for active EBV infection, such as anti-early antigen (anti-EA) IgM and IgA, were found in patients with relapses, but not in clinically stable patients [133]. Due to these findings, the authors suggested that disease activity in MS may be related to re-activation of EBV. Interestingly, a role for vitamin D can also be found in the response against EBV, as evidence shows that anti-EBNA-1 antibodies are reduced in patients receiving vitamin $D_3$ supplementation [134]. NK cells may be involved in EBV in multiple ways. Obviously, as major players in the innate viral immunity, NK cells play a role in the early defence against EBV infection. Indeed, an expansion of NK cells is seen in IM, which is correlated to lower viral load levels in some cases [135]. Likewise, depleting NK cells in mice results in failure to control EBV infection [136]. EBV infection even seems to elicit phenotype changes in NK cells, with an upregulation of NKG2A [137,138], but do not promote a memory-like NK cell phenotype [139]. Besides EBV infection, CMV infection also seems to induce a wide array of changes in the immune system, specifically targeting NK and T-cells [140,141]. For example, as mentioned earlier, CMV is of key importance in the formation of NKG2C + memory-like NK cells, which are more potent in their antibody-dependent mechanisms, but are more limited in their surveillance-dependent potency. In MS, CMV seems to play a protective role in its pathogenesis, as CMV seropositivity was associated with a decreased MS risk (OR = 0.73) [18]. The mechanism of protection is not yet fully understood, however, currently the protective effect of CMV is attributed to its immune configurative properties. For example, one theory states that the memory-like NK cells induced by CMV infection are especially potent in fighting viruses of the herpes family, including EBV [41,142].

### 4.3. Lifestyle

Lifestyle factors seem to play a role in the pathogenesis and disease progression of MS. Of these factors, the most prominent ones are smoking [143,144] and adolescent obesity [145–147]. So far, no specific component of tobacco smoke or adipose tissue has been linked to MS. Indeed, oral tobacco (snuff) actually seems protective against MS

[148]. The effect of smoking on NK cells has not been researched in the context of MS, but general studies regarding smoking and NK cells may provide clues how smoking affects MS specifically. Evidence is mainly found in COPD and lung cancer studies and is somewhat contradicting. Some studies point towards an enhanced NK cell response and promoted expression of IFN-γ due to smoking in murine NK cells [149], as well as increased activated circulating NK cells [150]. However, other studies report a lower circulating volume of NK cells due to smoking [151,152], as well as a lower IFN-γ and TNF-α expression and reduced cytotoxicity [153,154]. Some of these differences may be explained by presentation of data. Studies reporting an increase in NK cells mostly report a higher percentage of NK cells in smokers, which does not equal an absolute increase in NK cells. It could be possible that smoking reduces all lymphocytes, but it reduces other cells more than NK cells, thus increasing the percentage of NK cells in peripheral blood. However, although smoking clearly affects NK cells in multiple ways, no specific connection between alterations in the NK cell population and MS can currently be made. Obesity has been proven to negatively influence the immune response [155,156] and increase the risk of many disorders, including viral infections [157,158]. There is also evidence of alterations in the NK cell population as a result of obesity, like reduced cytotoxicity and cytokine production [159–161]. Additionally, the fact that vitamin D is fat soluble means that obesity directly impacts circulating vitamin D levels and could lead to a vitamin D deficiency. Again, no specific link can be established between MS and the effect of obesity on NK cells. While it is evident that obesity impacts NK cells and MS, a specific mechanistic relation is currently not feasible. However, both smoking and obesity are general inducers of a pro-inflammatory state. As such, seeing as there currently seems to be no mechanistic connection between smoking or obesity and MS, it seems that this shift towards a pro-inflammatory state caused by lifestyle factors simply lowers the threshold for auto-immunity. A combined theory could be formed with the EBV hypothesis, formulating that EBV infection in a pro-inflammatory environment due to smoking and obesity has a higher risk of causing auto-immunity.

## 5. Natural killer cells in therapies for multiple sclerosis

As mentioned earlier, much of our knowledge about the CD56$^{bright}$ NK cell in MS comes from studies regarding daclizumab. Studies regarding the efficacy of daclizumab showed a significant reduction in MRI lesions and clinical progression, with improvements up to 50 % compared to interferon beta-1a treatment [11–13,100]. Although the therapeutic effects were evident, serious concerns were raised regarding its safety profile. Hepatotoxicity was noted in the safety trials, causing authorities to restrict the prescription of daclizumab to only patients who did not respond to other treatments. After its approval, several cases of serious inflammatory brain disorders emerged, causing the suspension and recall of the drug [162]. As such, although CD56$^{bright}$ NK cells seem to have a promising protective and therapeutic potential, no DMTs currently focus on the NK cell population. However, although other drugs were not developed with the intent of influencing the NK cell population, they might have an effect there. Several therapies and their effect on NK cells are listed below.

### 5.1. Interferon-β-1b

Interferon-β-1b is one of the earliest therapies for MS. It has a plethora of effector mechanisms, including a reduction of MHC class II molecule expression, reduction of T-cell proliferation, lowered IFN-β production and reduced expression of adhesion molecules [163]. Interestingly, interferon-β-1b also seems to upregulate MHC class I expression in murine neuron models. As MHC class I is the main inhibitory ligand for CD56$^{dim}$ NK cells, it could be postulated that part of interferon-β-1b's immunoregulatory effect is due to reduction of cytotoxicity of the CD56$^{dim}$ NK cell population. Additionally, interferon-β-1b seems to alter the CD56$^{bright}$/CD56$^{dim}$ NK cell ratio by expanding the CD56$^{bright}$ population in peripheral blood [164], as well as altering the phenotype of the NK cell [165]. This phenotype change consisted of a downregulation of the inhibitory LILRB1 receptor (which binds MHC-I class molecules) and an upregulation of the inhibitory NKG2A receptor (which binds MHC class-I antigen E). Thus, it does not seem to alter the overall inhibition or activation of the NK cell, but rather the method of inhibition. The exact mechanism by which interferon-β-1b facilitates the expansion of CD56$^{bright}$ NK cells is unclear, but theories include a mobilisation from the lymph nodes to the peripheral blood. Although interferon-β-1b is proven to be effective in slowing disease progression and reducing clinical events, newer drugs are far more potent and have a better prognosis, resulting in a decline of popularity for interferon-β-1b.

### 5.2. Natalizumab

Natalizumab blocks very late antigen-4 (VLA-4), also known as α4β1-integrin, a receptor which is crucial in the migration of immune cells between tissues. VLA-4 binds with its counter-receptor, vascular cell adhesion molecule (VCAM)-1 to facilitate cell migration into the CNS. Natalizumabs intended mechanism of action was to block VLA-4 on T-cells and monocytes so they would be unable to migrate into the CNS and thus be unable to exert their inflammatory effects there. Interestingly, circulating NK cells are increased with natalizumab use [166,167], possibly reflecting a reduced capacity to migrate towards tissues, including the CNS. Indeed, NK cells also express VLA-4 and seem to prefer using a VLA-4 dependent mechanism to migrate into the CNS [89]. As mentioned earlier in this review, NK cells in the CSF are mainly of the CD56$^{bright}$ phenotype. Therefore, as CD56$^{bright}$ NK cells are generally considered as immunoprotective in MS, it would seem that natalizumab exerts both positive and negative effects on the immunological composition of the CNS. Supported by natalizumabs positive outcomes [168–170], the lack of T-cells in the CNS seems to outweigh the reduction of CD56$^{bright}$ NK cells.

### 5.3. Glatiramer acetate

Glatiramer acetate (GA) is a drug derived from four amino acids common in MBP (Glu, Ala, Lys, Tyr). It was originally designed as a synthetic antigen capable of inducing EAE. However an opposite effect was found where GA actually protected against EAE instead of inducing it [171]. The main mechanism of action seemed to be based on a shift from Th-1 cells to Th-2 cells and activation of Tregs [172,173]. It was demonstrated that monocytes under GA are less responsive to pro-inflammatory stimuli, secrete higher amounts of anti-inflammatory cytokine IL-10 and secrete lower amounts of the pro-inflammatory IL-12 [174]. There also seems to be a role for NK cells in this shift towards Th-2 cells. *In vitro* experiments show that GA enhances the cytolysis of NK cells against both immature and mature DCs, which are implied in the activation of Th-1 cells [175,176]. Killing immature DCs is part of the physiological repertoire of the NK cell and GA seems to amplify this by enhancing the interaction between NK cytotoxicity receptors and immature DCs [176]. Killing mature DCs is not a physiological mechanism of the NK cell, but seems to be caused by GA's ability to reduce MHC class I expression on DCs [176]. Thus, less inhibitory signals are received by the NK cells which causes it to kill the DCs. Furthermore, GA decreases the IFN-γ production of NK cells, but slightly increases the TNF-α production [176]. In stimulating cytotoxicity against both immature and mature DCs, GA ensures that Th-1 cells are not activated by antigen presentation. As such, it seems that NK cells are not directly enhanced or altered by GA, but they may be key players in clearing GA-altered DCs.

### 5.4. Dimethyl fumarate

Dimethyl fumarate (DMF) is a drug which alters many different immune cell populations, although its exact mechanisms are not fully elucidated. DMFs active metabolite, monomethyl fumarate (MMF), is proven to downregulate T- and B-cell responses through various mechanisms, such as induction of apoptosis, stimulation of Tregs and inhibition of migration to injured tissues [177]. Although the main focus of research has been the adaptive immune system, more recent evidence points towards an effect on the innate immune system as well. More specifically, a marked expansion of the $CD56^{bright}$ population is noted in patients treated with DMF [178–180]. Additionally, an increase in NK cell degranulation is reported [180]. Seeing as DMF seems to influence nearly every part of the immune system [177], it seems unlikely that the $CD56^{bright}$ NK cell expansion is the sole cause of the immunoprotective effect of DMF. However, it seems likely that the $CD56^{bright}$ population plays a role in the beneficial effects of DMF.

### 5.5. Fingolimod

Fingolimod (FTY720) is a sphingosine-1-phosphate (S1P) antagonist, with an intended effect of retaining autoreactive lymphocytes within the lymph nodes [181,182]. Its phosphorylated form binds to four of the five S1P receptors ($S1PR_{1,3,4,5}$), used in the egress of lymphocytes from the lymph nodes, with $S1PR_1$ being the most crucial for most lymphocytes [183]. Fingolimod causes a lymphopenia due to an accumulation of lymphocytes in the lymph nodes, although NK cells seem less affected than T- and B-cells. This may be due to the expression of $S1PR_5$ by NK cells, which is less affected by fingolimod than $S1PR_1$, thus giving NK cells an alternative method of egressing from lymph nodes. It should be noted that $CD56^{dim}$ NK cells have a relative overexpression of $S1PR_5$, while the $CD56^{bright}$ NK population expresses relatively more $S1PR_1$. Some studies suggest that absolute circulating NK cell numbers do not change under fingolimod treatment [184,185]. Nevertheless, the NK cell population does seem to be affected by fingolimod, as a marked reduction of circulating $CD56^{bright}$ NK cells without a loss of IFN-γ and TNF-α production is reported [186]. In the CSF, an expansion of the $CD56^{bright}$ subset is noted [187]. As such, it can be postulated that at least some of fingolimods effect is by enriching the $CD56^{bright}$ population in the CSF, thus creating a more immunoprotective environment.

### 5.6. Ocrelizumab

One of the more recent additions to the arsenal of DMTs is ocrelizumab, an anti-CD20 monoclonal antibody developed to deplete the B-cell population. It is quite similar to the more well-known rituximab, another anti-CD20 monoclonal antibody which is used in a variety of auto-immune diseases including neuromyelitis optica, a differential diagnosis of MS [188]. The fact that B-cells are involved in MS is supported by several findings. For one, B-cells are found in MS plaques [189] and in meningeal follicles [190]. Also, one of the diagnostic hallmarks of MS, oligoclonal bands in the CSF, is produced by B-cells [191]. Additionally, as noted earlier, nearly all hypotheses regarding EBV's mechanism of inducing MS involve the infected, immortalized B-cell. As such, there are many ways for the B-cell to potentially influence MS and B-cell depletion seems like a viable method of limiting MS activity. Indeed, its effectiveness is evident from its clinical trials, showing reduced relapse rates and fewer MRI lesions [192,193]. Also, it is currently the only drug to be approved for the treatment of primary progressive MS (PPMS) [194]. Ocrelizumabs intended mechanism is to block a specific epitope of CD20 (which is different than the one bound by rituximab). CD20 is expressed in the majority of B-cell lines, but not stem cells, pro-B cells and plasma cells [195]. Since plasma cells are the main producers of antibodies, antibody levels are not affected by ocrelizumab treatment [196]. Also, a small subset of T-cells (~6%) seems to express CD20 [197]. By binding to CD20, ocrelizumab causes the depletion of B-cells mainly by mediating ADCC against the target cell and for a small part by mediating complement dependent cytotoxicity (CDC) and apoptosis [10]. Although CD20 is not expressed on NK cells, they are still involved in ocrelizumabs mechanism since they play a role in ADCC. As such, through the depletion of B-cells, no novel antibodies are produced to coat target cells, which in turn renders the ADCC mechanism of NK cells ineffective.

## 6. Conclusions

Multiple sclerosis is a remarkably complex disease with a multitude of interacting factors contributing to its pathogenesis and course. The relatively recent interest in innate lymphoid cells has revealed new key players in the disease, with NK cells being especially interesting due to their therapeutic potential, as shown in the clinical effect of daclizumab. We reviewed not only the direct relation of NK cells with MS, but also their involvement in the well-known environmental risk factors associated with MS. Additionally, we reviewed the current therapies for RRMS and their effects on NK cells. First, the more specific division of NK cells into four subsets gives rise to new perspectives in how NK cells might influence MS. The characterisation of memory-like NK cells is particularly exciting, as it shows a way for NK cells to easily create a pro-inflammatory environment. As such, this finding might reveal an additional target for therapy. As the concept is relatively new, the role of these memory-like cells must be investigated further to determine their exact role in the pathogenesis of MS and in exacerbations. Another characteristic of NK cells that warrants further investigation is the expression of NK cell receptors. Particularly the changes a NK cell undergoes in response to different infections (*e.g.*, EBV or CMV infection) may play a role in the pathogenesis of MS, suggested by the protective effect of CMV infection. If a change in receptor expression is cause for a faulty immune response causing MS or its exacerbations, therapy blocking or altering the expression of these receptors may prove beneficial. Although NK cells are heavily implicated in MS exacerbations, as shown by Caruana et al. [104], much less clinical evidence exists for a role in the pathogenesis of MS. To explore the early diagnostic potential of NK cells, a study correlating NK cell counts and conversion from CIS to MS is necessary. With MS being a complex, multifactorial disorder, it is imperative to place the NK cell within the context of known environmental risk factors. As NK cells express a VDR, a direct link between NK cells and vitamin D levels can be established. It seems that vitamin D has an immunoregulatory effect on NK cells, thereby contributing to protection against MS. On the other hand, EBV is a known risk factor for MS and might even be a requirement for developing the disease. NK cells are the first line of defence against viruses and EBV seems to alter the NK cell phenotype. Smoking and obesity also contribute to the development of MS, but seem to do so in a non-specific way by stimulating a pro-inflammatory environment. Although no current DMTs specifically target NK cells, some therapies do influence the NK cell population. Mostly, this seems to consist of an increase in $CD56^{bright}$ NK cells, although none increase the $CD56^{bright}$ population as much as daclizumab did. Considering the undeniable therapeutic effects of daclizumab, the line of $CD56^{bright}$ NK cell enhancement as a therapy should be explored further, despite the initial setback of daclizumabs hazard profile. In conclusion, NK cells are established as key players in MS. The more we learn about the way they influence the disease, the more we can look towards using NK cells as a diagnostic or safe therapeutic tool in combating MS.

## Statement of interests

MM has nothing to disclose; JS received lecture and/or consultancy fees of Biogen, Merck, Sanofi-Genzyme, and Novartis; RH received institutional research grants and fees for lectures and advisory boards from Biogen, Merck, and Genzyme-Sanofi; JD has nothing to disclose.

## Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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## Appendix A. Supplementary data

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